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The goal of this project is to establish a foundation on which to develop a liposome-based platelet substitute in transfusion therapy. To this end, in the first year of this proposal, platelet membrane glycoproteins IIb and IIIa were isolated and incorporated into phospholipid vesicles as a heterodimer complex. Glycoprotein Ib was also purified by Triton solubilization of platelet membranes and affinity chromatography. The out-side out orientation of these glycoproteins in the liposomal vesicles, which were prepared by reverse-phase/sonication, was confirmed by antibody binding, ligand binding, and monoclonal antibody binding. In this grant period the expression of neoantigens on the surface of these liposomes was assessed. None could be detected by immune lysis assay in rabbit immunized with IIb-IIIa liposomes of soluble liposomes. GP IIb-IIIa liposomes did not activate humoral coagulation and did not induce platelet aggregation in vitro. In addition, no activation of coagulation in vivo could be detected. A model system was developed to evaluate the adhesive function of GP-liposomes in vitro under gravity and shear conditions. GP IIb-IIIa liposomes were shown

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to adhere effectively to de-endothelialized aorta and surfaces coated with vWF and fibrinogen. A thrombocytopenic rat model was developed to be utilized in in vivo studies of hemostatic efficacy of various liposome preparations in vivo. These studies show promise toward the development of a platelet substitute; however, a number of basic modifications of these liposomes are anticipated before an effective hemostatic moiety is achieved.

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FOREWORD

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Mary Ellen [illegible] 9/25/88

PI Signature Date

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Introduction

Platelets have a central role in hemostasis and have been implicated in pathologic processes such as atherosclerosis, myocardial infarction, stroke and tumor metastases (1-4). Platelet transfusions have assumed a critical role in the management of the thrombocytopenic patient and the patient requiring massive transfusions for the treatment of trauma. The last decade has seen major advances in the understanding of platelet physiology. Platelet membrane GPIIb and IIIa have been characterized as platelet membrane glycoproteins which form a Ca^{2+} dependent heterodimer complex (5-9) that belongs to a family of adhesive protein receptors, the integrins(10,11). During platelet activation, this complex undergoes a conformational change and functions as a fibrinogen receptor.(12-17). This fibrinogen binding is essential for platelet aggregation as demonstrated by the bleeding diathesis and absent platelet aggregation in Glanzman's thrombasthenia, an inherited deficiency of these GP,(18-21) and by the ability of a number of monoclonal antibodies to this complex to inhibit platelet aggregation (22). These proteins have been cloned(23-26), amino acid sequences determined, and some functional regions identified e.g.putative Ca^{2+} binding regions with similar structure to calmodulin (24,26). Fibrinogen binding properties of the soluble complex have been defined and purified complex inserted into liposomes (27-29) with preservation of fibrinogen binding. The proposed sites on the fibrinogen molecule that interact with the GPIIb-IIIa complex are a dodecapeptide (His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val) (His-12-Val), residues 400-411 of the carboxyl terminus of the fibrinogen gamma chain, (30-32) and a tetrapeptide (Arg-Gly-Asp-Ser:RGDS) near the carboxyl terminus of the alpha chain or a tripeptide (Arg-Gly-Asp: RGD) near the amino terminus of the alpha chain (33-37). The synthetic peptide analogues of these regions inhibit platelet aggregation and fibrinogen binding to activated platelets and are mutually inhibitory in binding studies (33-37). The data suggest that these peptides may share common or closely related binding sites on GPIIb-IIIa. RGDS is also a proposed sequence by which von Willebrand factor binds to GP IIb-IIIa and to GPIb.

Ligand binding to GPIIb-IIIa may signal secondary events in the platelet and alter the conformation and distribution of the IIb-IIIa complex. Isenberg et al have reported that binding of fibrinogen, RGDS, or the gamma dodecapeptide induces clustering of platelet IIb-IIIa (38). Banga et al have reported that occupancy of GP IIb-IIIa by fibrinogen is necessary to maintain Na^{+}/H^{+} exchange in epinephrine stimulated platelets (39). Parise et al have demonstrated that binding of LGGAKQAGDV or RGDS to soluble IIb-IIIa alters the hydrodynamic properties of the complex and renders GPIIb susceptible to thrombin hydrolysis (40).

Platelet membrane GPIb has also been extensively characterized. This glycoprotein contains a receptor for von

Willebrand factor(vWF). Antibodies directed against GPIb prevent vWF binding to the platelet surface and inhibit vWF-dependent ristocetin-induced platelet agglutination(42). This GP exists in the membrane in complex with GPIX, a complex which can remain associated following detergent solubili- zation of the platelet membrane(43). In addition, GPIb has been reported to be the receptor for quinine/quinidine drug dependent antibodies(44) and to bind thrombin. Thrombin and vWF receptor function may partially reside on the glycocalicin portion of Ib, a protease sensitive region(45). Berndt(46) and Canfield(47) have reported methods for purifying GPIb and GPIb-IX free of contaminating proteins. These purified proteins have been bound to solid substrate (48) and, partially purified proteins, incorporated into liposomes(49)to study their function. The amino acid sequence of GPIb has been obtained by cDNA (50,51) GpIb is heavily glycosylated but the role of carbohydrate in functional properties is not known.

In addition to platelet GP, platelet cytoplasmic ionized calcium concentration ($[Ca^{2+}]_i$) plays a central role in platelet activation. Several platelet agonists, such as thrombin, ADP, and platelet activating factor produce an increase in platelet $[Ca^{2+}]_i$ (52-56) which occurs on a subsecond time scale. Most of this Ca^{2+} is derived from intracellular sites such as the dense tubular system, although activation is also associated with increased platelet plasma membrane permeability to Ca^{2+} (57,58).

The role of extracellular calcium in human platelet aggregation has not been precisely defined. However, even in unstimulated platelets, in which the Ca^{2+} concentration is 10,000 fold less than plasma, Ca^{2+} exchange can occur across the plasma membrane. The potential role of the glycoprotein IIb-IIIa complex in platelet Ca^{2+} flux is supported by data that the maximum rate of platelet plasma membrane $^{45}Ca^{2+}$ exchange in thrombasthenic platelets is half that observed in normal platelets (59,60) and that dissociation of the IIb-IIIa complex in normal platelets causes a decrease in basal $^{45}Ca^{2+}$ influx. Studies with monoclonal antibody and a tetrapeptide ligand by Yamaguchi et al(61) and our studies with GPIIb-IIIa incorporated into liposomes(62) provide additional evidence that this glycoprotein complex may play a role in platelet Ca^{2+} flux. However, from monoclonal antibody studies on the intact platelet, Powling et al have suggested that a platelet plasma membrane calcium channel is located in proximity to the GPIIb-IIIa complex but postulated that it is not the GPIIb-IIIa complex (63).

A number of key questions remain concerning platelet physiology. These include further details of the conversion of reversible platelet aggregate to an irreversible aggregate, the role of transmembrane Ca^{2+} flux during platelet activation and the precise role of IIb-IIIa in platelet Ca^{2+} flux, the contribution of GPIIb-IIIa to platelet adhesion to a wound surface and the biochemical nature of these adhesive interactions. Enhanced understanding of platelet physiology will facilitate development of platelet substitutes to be used in transfusion therapy. The latter assume greater importance daily due to increasing use of platelet transfusions, the relative shortage of platelet

concentrates and the risk of transmission of infectious disease by such concentrates.

The goal of the current proposal is to define a basic foundation for the development of a phospholipid vesicle (liposome) with platelet membrane glycoproteins integrated into the surface, as the basis for such a platelet substitute. The first phase of this goal is to address critical issues of feasibility.

Approach

The general schema of the 2 year proposal is outlined in table 1.

Liposome preparation

A. Protein preparation: 1. GPIIb-IIIa; 2. GPIb

IIb-IIIa Preparation:

Glycoproteins IIb and IIIa were prepared as previously reported (62,65) from Triton X-114 (Sigma Chemical, St. Louis, Mo) solubilized platelet membranes. Clinically outdated platelet concentrates were washed x 2 in citric acid buffer (0.013M citric acid, 0.013M sodium citrate, 0.033M D-Glucose, 0.15M NaCl, pH7.0) and x 2 in Tris buffer (0.01M Tris, 0.15M NaCl, pH 7.24). Washed platelets were resuspended in Tris buffer (0.01M) pH7.4 with 0.5mM $CaCl_2$, 0.15M NaCl with phenylmethylsulfonylfluoride (PMSF) (0.4mM) and leupeptin (100ug/ml). Platelets were sonicated on ice and centrifuged (1000xg) to remove non-disrupted platelets. The supernatant was centrifuged at 78,000 xg for 1 hr and the pellet suspended in 1% (V/V) precondensed Triton X-114, 10mM Tris, 0.15M NaCl, pH 7.4 with 0.4mM PMSF, incubated overnight at 4°, and centrifuged at 78,000xg for 1 hr at 4°C. The detergent phase was applied to a 6% sucrose cushion, heated at 37° for 5 minutes, centrifuged at 1500xg for 5 minutes, and the detergent micelle aggregate layer removed. Further purification of proteins was achieved by lentil-lectin Sepharose chromatography, with 10% alpha-methyl-D-mannoside elution. Detergent was partially removed with Biobeads SM2. Each IIb-IIIa preparation was assayed for total protein by modified Lowry (66) and residual Triton X-114 determined spectrophotometrically (A_{622}) after reaction with ammonium cobaltothiocyanate reagent and solubilization in ethylene chloride (67). Preparations contained up to 0.27mg Triton per mg protein. Previous data with dissociated GPIIb and IIIa demonstrated that this residual detergent did not alter the experimental results. Proteins were analyzed by SDS-PAGE, reduced and non-reduced, using 7.5% gels. While small amounts of contaminating proteins theoretically may have been present, they were not detectable by combined silver and Comassie blue stains of gels of any protein preparation used. SDS-PAGE of the proteoliposome preparations revealed only 2 protein bands(Figure 1).

GPIb preparation

Glycoprotein Ib was prepared from platelet membranes by a

modification of the method of Canfield et al(47). Briefly, platelets were washed 3 times at 22°C in 10 mMTris buffer pH7.4, 140mM NaCl, 1mM EDTA (TSE). Washed platelets were be suspended in 10mMTris, pH8.1, 140mM NaCl, 10mM EDTA, 2mM N-ethylmaleimide (NEM), 2mM phenylmethylsulfonylfluoride, and 0.1mM leupeptin. Protease inhibitors and NEM are required to inhibit protease activity which would result in free glycocalicin. NEM also disrupts the interaction between the GPIb complex and the membrane skeleton. Platelets were sonicated in a Fisher Sonic Dismembrator Model 150 (10sec x 6 at 3.5 output with microtip) at 4°C, centrifuged (10,000g,15 min. 4°C); deoxycholate(DOC) (final 0.5%), was then added to the supernatant. The suspension was centrifuged (10000g, 60min). The resulting supernatant was applied to a wheat germ agglutinin-Sepharose column; the column washed with TSE pH8.1 with 0.1%DOC and TSE without DOC. The column was eluted with TSE plus 2.5% N-acetyglucosamine without DOC, and TSE, NAG and 0.1%DOC. GPIb eluted in the DOC buffer. This eluate was dialyzed against 10mMTris, 140mM NaCl. Potential contaminating GPIIb-IIIa were removed by anti-IIb-IIIa immuno-affinity chromatography. These anti-IIb-IIIa antibodies were generated in our laboratory as a component of the rabbit immunization experiments describe below. Proteins were analyzed by SDS-PAGE. Total protein was quantitated by the method of Lowry (66) and GPIb concentration by ELISA. This method has been shown to minimize proteolysis and to enrich for GPIB. An alternative approach is GPIb production by Triton solubilization of platelet membranes and immunoaffinity chromatography to isolate GPIb-IX and Ib(46). The limitation of this alternative is loss of activity during elution from the affinity column. This method was tried using commercial (Chemicon CO) anti-Ib antibody but was subsequently dismissed as a viable alternative.

B. Proteoliposome Preparation

Large unilammelar vesicles were prepared from phosphatidylcholine(PC) (Type IVe; egg yolk, 99% pure, Sigma Chem Co) by the reverse-phase method as previously described (62,65) Briefly, PC (25mg in hexane at 100mg/ml) was dried to a thin film on the bottom of a 50ml round bottom flask in vacuo, 3-14 hours. The lipid film was resuspended in 1.5ml of ether and then 0.25ml of 10mM Hepes, 0.150mM NaCl, pH7.4. This mixture was sonicated for 2 min in a bath type sonicator. The ether was removed by rotary evaporation under reduced pressure to form a lipidic gel. This gel was then sonicated for 1 min with 1 ml of buffer containing glycoproteins (100ug/ml). Liposomes were washed x 2 by centrifugation at 14,000g for 10 min at 4°C. Liposomes prepared by this method are relatively homogenous in size and entrapped volume, with mean diameter of 5u by laser light scattering with a Coulter N4 Submicron Particle Sizer. On average, 70% of added protein is incorporated, with 48.6 $\pm$ 0.8% of protein in an outside-out orientation as determined by vibrio cholera neuraminidase cleavage of sialic acid residues. As previously reported, GPIIb-IIIa complex incorporated onto the phosphatidylcholine liposome surface binds fibrinogen with approximate $Kd=10^{-7}$ (27-29) and bound monoclonal antibodies

against GP Ib-IIIa (27). SDS-PAGE of proteins associated with SDS-solubilized liposome preparations, revealed only 2 bands consistent with IIb and IIIa (18).

C. Immunogenicity

Expression of neoantigens when platelet glycoproteins are inserted into a new lipid environment is an important issue for the use of a liposome based moiety as a tranfusion modality. This has been addressed by immunization of rabbits with: liposomes, liposomes bearing IIb-IIIa on the surface, solubilized IIb-IIIa, and activated platelets. Antibody response was then compared by immune lysis assay. Specifically, the ability of immune sera for sets of animals immunized with the moieties described above were compared for their ability to lyse target IIb-IIIa liposomes and simple phosphatidylcholine liposomes loaded with glucose. Sera were then compared after extensive adsorbtion with human platelets. The hypothesis in this system is that neoantigens specific to glycoproteins incorporated onto the surface of liposomes will not be removed by this adsorbtion procedure. Prior to testing, sera were absorbed with bromolinated human red blood cells to remove anti-human antibodies.

Female (6lb) New Zealand White rabbits were immunized (2 animals per immunogen). Prior to immunization, rabbits were observed for 1 week and preimmunization sera were obtained from an arterial puncture to determine presence of natural antibodies. The immunization procedure involved one injection into the popliteal lymph node, exposed by surgical dissection under anesthesia. For this procedure, animals were anesthetized with I.M. Ketamine(35mg/kg) and Rompun(5mg/kg). Evans blue dye was injected between the toes to facilitate visualization of the lymph node. This method has been shown to enhance immune response to small amounts of antigen. Antigens injected included: phosphatidylcholine liposomes (12.5mg lipid), IIb-IIIa liposomes (100ug protein in 12.5mg lipid)),or solubilized GpIIb-IIIa (100ug). All antigens were emulsified in complete Freund's adjuvant (Liposomal integrity was preserved) (68,69). Additional rabbits received intravenous injections of soluble IIb-IIIa or intact human platelets (100,000/ul). Three subcutaneous boosts were given at 3 week intervals with antigens emulsified in incomplete Freund's adjuvant or rabbits which had received intravenous primary immunization, received intravenous boosts. Rabbits were bled every 7-10 days and sera tested by immune lysis assay.

The Immune Lysis Assay utilized a modification of the glucose release method of Kinsky (70,71). Before testing, antisera were heated at 56°C for 30 min. to inactivate complement and then were centrifuged at 27,000g for 1hr. to remove aggregates and low density lipid. Antisera were absorbed with bromolinated human RBCs to remove anti-human antibodies, and, in selected experiments, with human platelets. Fresh human sera were used as a source of complement. All sera were extensively dialyzed to remove glucose. Sera were frozen at -70°C in aliquots until testing.

Glucose release assay reagent: both complete assay reagent

(CAR) and incomplete assay reagent (IAR) were prepared in Tris buffer pH7.5 with NaCl, $MgCl_2$, $CaCl_2$ with hexokinase(30ug/ml Boehringer-Mannheim, dialyzed to remove ammonium sulfate), glucose-6-phosphate dehydrogenase (15ug/ml, dialyzed), ATP (92mM), NADP(TPN)(1mM) for CAR. IAR did not include NADP, therefore is used as a blank. The assay reagent was freshly prepared daily.

Liposomes were prepared as described above but with 0.3M entrapped glucose. Prior to use, liposomes were washed to remove untrapped glucose. The amount of residual untrapped glucose was measured as described below.

The final assay mixture included: 0.5ml glucose assay reagent(CAR or ICAR), 0.355ml of 0.15MNaCl, 0.2ml of antiserum to be tested, 0.12ml complement and 0.005ml of liposomes (control or IIb-IIIa).

Two A340 readings were recorded: pre and 30 min post addition of liposmes.

To measure total glucose, duplicate test tubes were set up containing 0.005ml of liposomes and 0.5ml of chloroform (to release entrapped glucose;removed by a stream of N_2) and either CAR or ICAR and buffer added. The $A340_{CAR}$ minus $A340_{ICAR}$ (background) equals total glucose. Untrapped glucose was measured in a similar manner without the addition of chloroform, with correction for liposome absorbance.

The glucose released by the test serum is expressed as per cent trapped glucose released :

$$= \frac{\text{A340 final assay mixture(CAR)-background}}{\text{A340 trapped glucose}}$$

$$= \frac{\text{A340 final assay mixture(CAR)-A340 final mix(ICAR)}}{\text{A340 chloroform releasable-A340 untrapped}}$$

Adhesion

Proteoliposomes were preparaed by reverse-phase/sonication as described above. For smaller liposomes,(200-900A), an additional step of high pressure extrusion by French Press was added(72,73). An alternative approach for smaller liposomes, is extensive sonication; however, this denatures the incorporated protein. Liposomes were sized by Coulter N4 Submicron Particle Sizer and were homogeneous. For radiolabelling of lioposomes, ^{3}H-phosphatidlycholine (New England Nuclear) was used in the preparation.

Non-shear adhesion was performed in microtiter wells by a modification of the method of Pytella et al (10). ^{3}H-liposome suspensions (IIb-IIIa, control, GPIb, composite) were allowed to settle on microliter wells (Linbro 96 flat bottom wells 1.0x0.6 cm, Flow Laboratories Inc.) precoated with BSA or the purified substance of interest (Collagen, FGN, FNC) plus BSA (to block residual sites), or containing circlets (10-50mm diameter) of de-endo- thelialized bovine or human (2hrs post mortem) aorta with and without cryoprecipitate. After incubation, the wells were gently washed, fluor added, and ^{3}H counted in a beta

counter. All experiments were performed with at least 10 replicates and repeated at least 3 times in order to attain statistical significance. At this point in the study, GPIb and composite liposomes have undergone only preliminary studies due to the difficulty is attaining pure, functional Ib.

A method to measure adhesion under shear stress was developed using a polymethacrylate chamber modified from Sakariassen et al (72). The flow in this chamber enters in a cylindrical tube with the inlet oriented at a 20° angle to a rectangular cross section with a depth of 0.195+ 0.005mm and width 18mm (to contain coverslips 18x18x0.147+ 0.003mm or thin tissue sections) with a removable central knob. The inlet is connected to an injection syringe for controlled-rate injection. Coverslips can be coated by air-gun with delipidized BSA or the purified substance of interest (Collagen, vWF, FGN, FNC) plus BSA (to block residual sites) and cultured fibroblast monolayers or thin tissue sections or de-endothelized human(post-mortem) and bovine aorta in the chamber. Liposomes (control, IIb-IIIa, Ib,composite) suspensions can be infused through this system and adherent liposome-^{3}H counted in a scintillation counter and total adherent lipid quantitated.

Effect on coagulation in vitro and in vivo

Due to concern over the possibility of massive activtion of platelets or of humoral coagulation in vivo by liposomes bearing platelet membrane proteins, our liposomes were assessed in stndard in vitro assays--prothrombin time(PT) and partial thromboplastin time(PTT) using automated techniques with kaolin and thromboplastin reagent as activators; the whole blood clotting time was maesured by mechanical resistance. Platelet aggregation (spontaneous, collagen0.8-16ug/ml, ADP 1.6-32uM, epinephrine5-40uMand arachidonic acid200-500uM induced) was measured with a dual channel aggregometer. Blood was anticoagulated with Na citrate(0.011M) and platelet rich plasma(PRP) prepared by centrifugation(15 Min,100g). Spontaneous and agonist induced aggregation with and without liposomes was measured. Initial rate of aggregation and degree of responsiveness were measured. To study in vivo effects, fibrinogen concentration and platelet counts were obtained in rabbits following intravenous administration of control and specific liposomes.

Results

Protein orientation on the surface of the liposomes. We have shown that GP IIb-IIIa liposomes prepared by reverse-phase bind monoclonal antibodies directed against GP IIb/IIIa (Table 2). The ability to insert these proteins in an out-side out orientation and for these proteins to remain as a hetero-dimer complex is an essential prerequisite to a potential platelet substitute. This has been achieved for GPIIb-IIIa and preliminary data sugges that this is also feasible for GPIb.

GPIIb-IIIa on the surface of liposomes bound ^{125}I-fibrinogen with a Kd=10^{-7} and binding isotherm shown in figure 1. While this observation confirms the integrity of at least some of the complexes in the surface of the liposomes, it could prove to be problematic in localization of liposomes to a wound surface if these receptors are blocked with bound ligand.

GP IIb-IIIa were shown to mediate Ca^{2+} and fibrinogen dependent liposome aggregation. The characteristics of these aggregates are detailed in Table 3. Aggregation was inhibited by monoclonal antibody to the IIb-IIIa complex. Importantly for potential in vivo use, these aggregates did not occur spontaneously but required induction by centrifugation. Aggregate formation does confirm the possibility of inter-liposomal interactions to be mediated by glycoproteins. However, these aggregates were highly susceptible to disruption by shear forces; further modifications will be necessary for stable interactions to occur at a wound site.

Liposome stability Stability of liposomes in buffer and in plasma were determined by use of encapsulated carboxyfluorescene. Due to the self-quenching property of this compound, release of intra-liposomal contents can be monitored over time. In addition, liposome size was montored; change is size is an index of rupture and fusion.

GpIIb-IIIa liposomes were found to be stable in buffer for 7 days and in plasma for 10-14 days. The results were identical for phosphatidylcholine and phosphatidylcholine:cholesterol (70:30-mole:mole) liposomes. These results are shown in tables 4 and 5.

Activation of coagulation and of platelets GPIIb-IIIa liposomes had no significant effect on in vitro coagulation. These results are shown in table 6. Proteo-liposomes were able to participate in platelet aggrgate formation, as determined by association of ^{3H}-GpIIb-IIIa liposomes but not control liposomes with activated platelets but did not induce spontaneous aggregation nor change the initial rate or final respnsiveness of platelets to agonists(table 7).

For in vivo assessment, animals were followed for the activation of coagulation after intravenous administration of GPIIb-IIIa liposomes. There was no evidence of DIC, specifically platelet count and fibrinogen levels were unchanged (platelets/ul: pre:199,355; post: 167,445 for GPIIb-IIIa

liposomes; pre 195,024; post:116,388 for control; fibrinogen mg/dl-- pre 680+ 45, post 610+ 50 for GPIIB-IIIa liposomes; no significant difference).

Adhesion Experiments were performed to assess the adhesion of ^{3}H-PC liposomes (control and IIb-IIIa) to de-endothelialized bovine and human aorta, and to microtiter wells. A 200ul suspension of ^{3}H-liposomes (100-200x10^{3} cpm, 12ug lipid) was added to bovine or human aortic segments, which had been gently scraped to ensure deendothelialization, or to microtiter wells which were pre-incubated with buffer, cryoprecipitate, collagen, fibrinogen, or fibronectin. Liposomes were preincubated with buffer or plasma. Following incubation at 25°C, aortic segments or wells were gently washed and adhesion quantitated by mesurement of bound counts. The amount of bound lipid was then calculated. These data are shown in Table 8. In these studies, IIb-IIIa liposomes required pre-coating of aortic segments with cryoprecipitate (presumably fibrinogen or Von Willebrand) for adhesion to occur. Pre-incubation with plasma, inhibited adhesion, possibly by fibrinogen or von Willebrand receptor blockade.

These data, which obviously require further study, do suggest that this is a useful approach by which to study the adhesive properties of IIb-IIIa and Ib. In addition, similar results were obtained with coated coverslips placed in a flow chamber modified from Sakariassien (72) detailed in experimental methods. As anticipated, with shear stress, less adhesion was observed. These studies are still in progress. It is anticipated that GPIb will facilitate adhesion, particularly in the plasma milieu beacause this GP does not bind soluble von Willebrand factor or fibrinogen and thus will not be susceptible to receptor blockade.

Thrombocytopenic rat model. Data indicate that external total body irradiation (TBI) by Cesium source is an effective modality with which to induce thrombocytopenia in rats. Male, Sprague-Dawley out-bred rats (400g), after 2 weeks of observation, were given 0 (control), 200, 400, 600, 900 Rads TBI by Cesium unit. Platelet counts were then assessed on days 0-14 by amputation of a 0.5cm segment from the tail and collection of 50ul of blood in EDTA (0.2% final). Platelet counts were performed in a Baker series 810 Platelet Analyzer with thresholds set for rat platelets ($1.7u^{3}$-$18u^{3}$). By day 10, the 900 Rad TBI group had a platelet count <20,000/ul and remained thrombocytopenic until expiration (day 14). Representative platelet counts are shown in Table 9.

Immunization Spontaneous anti-lipid antibodies were detected in rabbits but not in the humans tested (Table 10). Soluble GPIIb-IIIa, when administered intravenously or by popliteal lymph node injection, were highly immunogenic in rabbits as anticipated. Rabbits also developed increasing titers of anti-lipid anti-bodies which is characteristic of that species. The time course of antibody development as determined by immune

lysis assay is shown in figure 3. GpIIb-IIIa liposomes administered into the popliteal lymph node were more immunogenic than intravenously administered liposomes and induced a more rapid immune response than soluble GPIIb-IIIa. These antibodies reacted with GPIIb and IIIa by Western blot analysis. This immune response was anticipated in rabbits (in contrast to humans) since these are foreign proteins. Importantly, the antibody could be completely adsorbed by human platelets (figure 4). This suggests that neoantigens are not expressed by the purification of GPIIb-IIIa and insertion into a phosphatidyl-choline bilayer.

GPIb preparation By DOC solubilization of platelet membranes and a combination affinity chromatography columns, 100ug of GPIb per 100 units of clinically out-dated human platelets is obtainable. This glycoprotein is currently under study. Initial data suggest that functional activity is preserved.

In summary our first year studies demonstrate:

a. Feasibility of incorporation of GP IIb-IIIa into liposomes with retention of reactivity with monoclonal antibodies, and fibrinogen binding
b. Ability of GP IIb-IIIa incorporated onto the surface of liposomes to mediate fribrinogen and Ca^{2+}, or cryoprecipitate dependent liposome aggregation
c. Proteo-liposome stability in plasma up to 14 days
d. Ability of GPIIb-IIIa liposomes to adhere to de-endothelialized bovine and human aorta in the presence of bound cryoprecipitate components
e. Feasibilty of the modification the adhesion chamber of Sakariassesn for use with 1 ml samples of liposome suspensions.
f. Lack of detectable neoantigen exposure on glycoproteins IIb and IIIa by insertion into a new lipid milieu.
g. Lack of spontaneous activation of coagulation or of platelets by GPIIB-IIIa liposomes.
h. Ability to purify glycoprotein Ib in quantities sufficient for study in liposomes.
i. Ability to induce thrombocytopenia in Sprague-Dawley rats with external Cesium radiation while maintaining animal viability, in order to use this model to study the efficacy of various liposome preparations in vivo.

Discussion

The complex nature of platlet physiology makes the development of an artificial platelet a formidable task. In this project, a determination of the minimum requirements for a platelet substitute, which would have some of the properties of a platelet, and the feasibilty of a liposome based approach for

this substitute are being evaluated. To this end, we have determined that platelet membrane glycoproteins can be successfully incorporated into an artificial membrane in a functionally active configuration. In this model, GPIIb-IIIa-liposomes can adhere to subendothelium under non-shear conditions. A model for study of shear conditions has been developed; initial data suggest that, with shear, GPIIb-IIIa liposome adhesion is less than under non-shear conditions. Adhesion is one of the minimum requirements for a platelet substitute. In addition, GPIIb-IIIa on a liposome surface can bind appropriate ligand, another essential requirement for a platelet substitute. However, the data indicate that plasma inhibits both of these activities. Thus, as anticipated, additional surface constituents will be necessary to facilitate adhesion at a wound surface. GPIb is a promising candidate since this GP does not bind soluble ligand. We have developed a procedure for purification of active GPIb in sufficient, albeit still limited, quantities for evaluation in liposomes. The characteristics of this GP in liposomes and of composite liposomes (GPIIb-IIIa-Ib) are currently under study.

An important consideration in the development of a liposome based platelet substitute is the possibility of the exposure of new antigens by the purification and liposome insertion of glycoproteins. The data from rabbits immunized with soluble GPIIb-IIIa, liposomal GPIIb-IIIa, and human platelets suggest that new antigens do not emerge for these GP. However, when an effective substitute is developed, the final determination must be made in the clinical situation, analogous to the administration of monoclonal antibodies and recombinant proteins to patients. Nevertheless, the current data are promising.

Proteo-liposome stability in plasma is another important consideration. The reverse-phase methodology generates proteo-liposomes which are stable in plasma for up to 14 days. This is adequate for clinical administration; liposomes will be cleared by the reticulo-endothelial system before inherent stability problems emerge and would, ideally, rapidly adhere to a wound site. The stability observed with this preparation both in plasma and in buffer is inadequate for long term storage of an effective moiety. Thus preservation methods such as lyophilization will be necessary.

Another consideration for a platelet substitute is the possiblity of massive activation of coagulation in vivo following administration. In vitro and in vivo (rabbit) evaluation of proteo-liposome preparations to this point have shown no evidence of such activation.

In order to assess the efficacy of a platelet substitute, an in vivo model is essential. Such a model requires integrity of humoral coagulation, thrombocytopenia, and a method of evaluation of primary (platelet) hemostasis. The rat tail bleeding time, under carefully controlled conditons, in the irradiated thromboctytopenic rat is such a model. We have

demoonstrated the ability to induce consistent, prolonged thrombocytopenia in rats with Cesium irradiation which preserves humoral coagulation, in contrast to exchange transfusion which requires plasma administration to compensate for coagulation factor depletion, and to other thrombocytopenia models which require the administration of anti-platelet antibodies which would also effect proteo-liposomes. The tail vein bleeding time in these animals will provide an index of efficacy of a platelet substitute. This model will provide a primary screen for efficacy; however, a model, representative of the trauma situation will be required once an efficacious substitute is developed.

Conclusions and future directions

The first year of this project has achieved stated goals toward developing a platelet substitute. In the next year further modifications will be made in our "plateletsomes". These include the study of the in vitro properties of GPIb and composite liposomes, the study of proteoliposomes in vivo, and the effect of alteration of membrane lipids on function.

A promising lipoosome surface constituent to be considered, in the future, is the platelet collagen receptor which has recently been purified and incorporated into liposomes. In addition, platelet glycoproteins produced by recombinant technology which could potentailly overcome several of the pitfalls and limitations of purification of proteins from platelets will need to be evaluated functionally in liposomes. Monoclonal antibodies to subendothelial constituents could also facilitate liposome targeting to a wound and should be evaluated in this model.

Thus, while significant obstacles exist, a number of potentially fruitful avenues are emerging for the ultimate goal of developing a platelet substitute.

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Figure 1

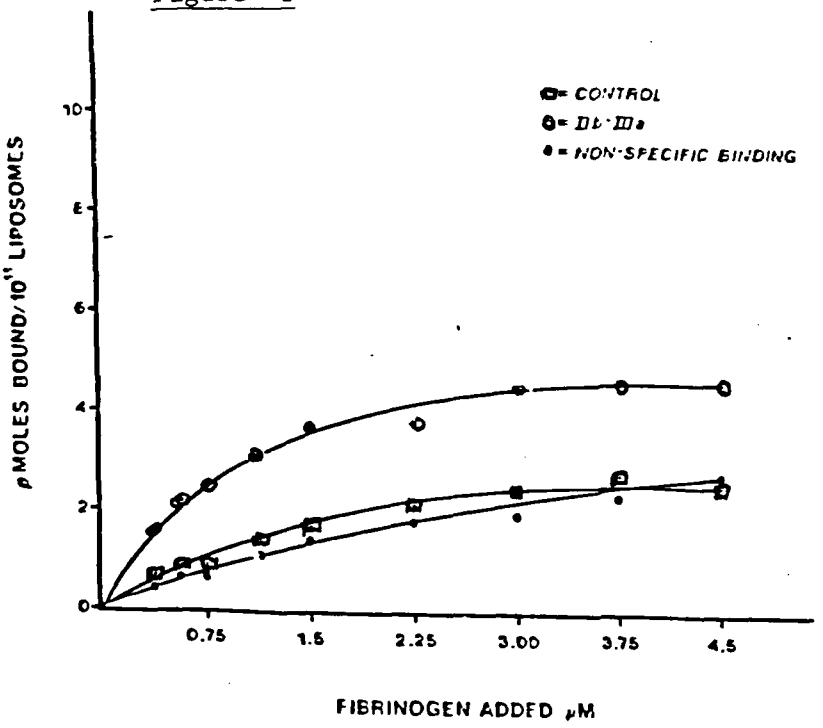


Fig. 2 ^{125}I-fibrinogen binding to IIb-IIIa liposomes. 250 ul of liposomes (1×10^5 liposomes/ul) of control liposomes or IIb-IIIa liposomes were incubated with ^{125}I-fibrinogen for 30 min at 37° then washed in 4% sucrose with centrifugation at 10,000 xg. Non-specific binding was defined as binding in the presence of 10 mM cold fibrinogen (●—●). Specific IIb-IIIa liposomes fibrinogen binding (○—○) is the net difference of total and non-specific binding. Control liposomes manifest only non-specific binding (□—□)

Figure 2

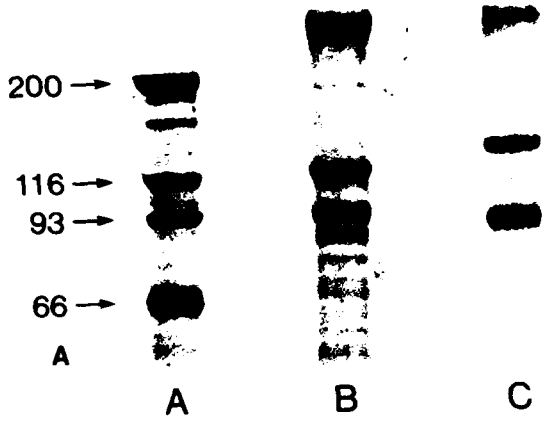
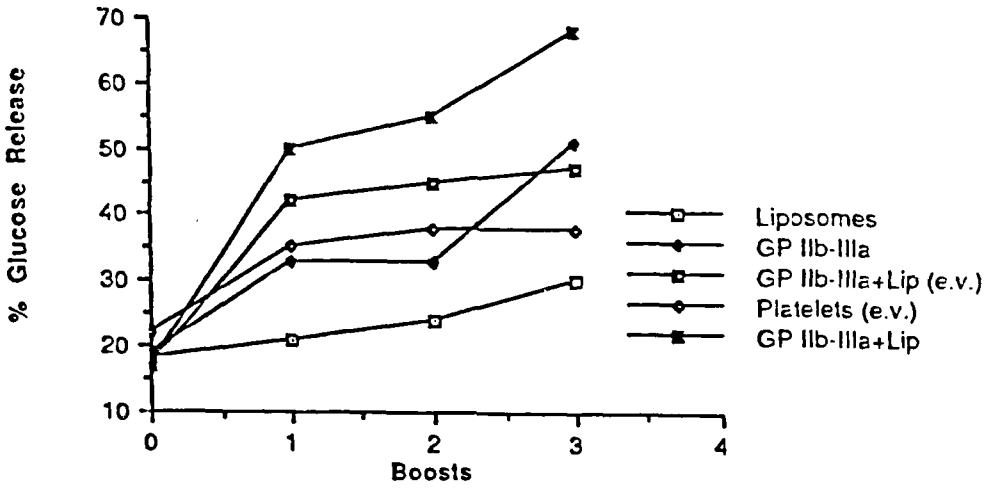


Fig 1. Nickel-stained SDS-PAGE (5% stacking, 7.5% running gel) of glycoprotein preparation before liposome incorporation. Molecular weight standards are shown in the first lane; in the second (B) and third (C) lanes, a IIb/IIIa preparation reduced with 2% dithiothreitol and unreduced are shown, respectively.

Figure 3

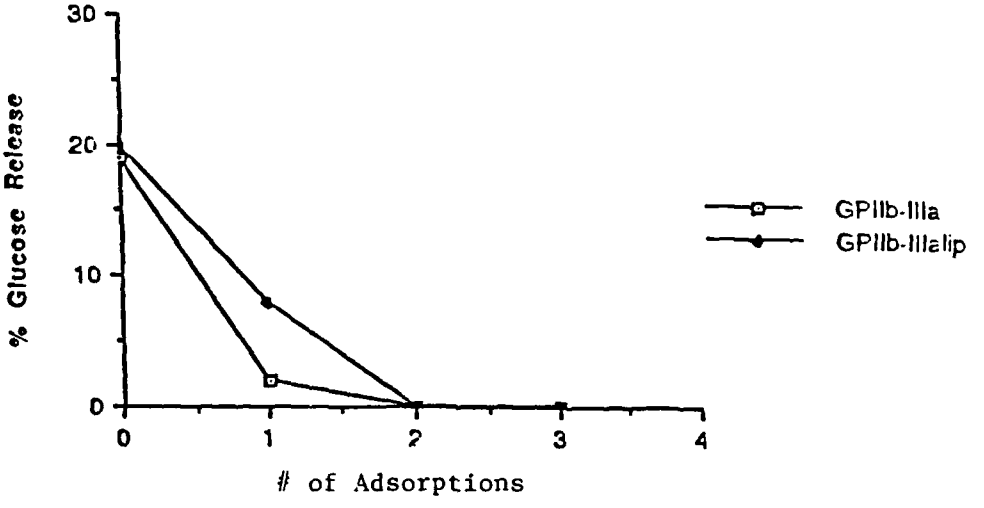
Release of Glucose from Liposomes by Rabbit Sera from Animals Immunized with Different Immunogens



Legend: Immune Lysis Assay was performed on rabbit sera adsorbed with human RBC, at the time points indicated, after immunization with the immunogen noted.

Figure 4

Immune Lysis Assay Activity after
Adsorption with Human Platelets



Legend: Residual activity in Immune Lysis Assay after adsorption with human platelets, for sera from rabbits immunized with soluble GPIIb-IIIa or with GPIIb-IIIa liposomes.

Table 1

Outline of revised proposal

Section 1 Theoretical Considerations and Experimental Plan

I. Preparation of liposomes which bind to components of subendothelium exposed at a wound surface.
    A. Proteins to be assessed
        a. Glycoproteins IIb and IIIa
        b. Glycoprotein I complex
        c. Combination of a and b
    B. Stability of proteins in membranes
    C. Protein-protein interactions
II. Effects of lipid composition on protein function
III. Effects of protein orientation on protein function
IV. Immunogenicity
V. Massive activation of coagulation in vivo

Section 2 Methods

I. Glycoprotein purification
    A. GPIIb-IIIa
    B. GPI

II. Plateletsome preparation
    A. Primary method
    B. French press modification
    C. Lipid composition
    D. Stability

III. Activation of coagulation by plateletsomes
    A. Effect on humoral coagulation
        1. in vitro
        2. in vivo
    B. Effect on platelet aggregation
        1. in vitro
        2. in vivo

IV. Immune lysis assay

V. Adhesion Studies
    A. Microtiter wells
    B. Immobilized collagen
    C. Denuded aorta

VI. In vivo hemostasis studies
    A. Rat
    B. Rabbit

Table 2

Binding of MoAbs to GPIIb-IIIa liposomes by total FITC-fluorescence (λ_{ex} 495nm, λ_{em} 525nm) of liposomes after incubation with specific monoclonal antibodies and irrelevant antibody plus FITC coupled anti-murine antibody or FITC anti-murine antibody alone.

Antibody	F	Source
M148	35.8 ± 5.1	Hardisty
7E3	27.6 ± 4.0	Coller
10E5	19.4 ± 3.1	Coller
AP2	21.4 ± 2.1	Kunicki
357 (irrelevant antibody)	13.2 ± 1.0	Herrman
FITC antimurine	15.5 ± 1.2	

Specific antibody fluorescence compared to irrelevant antibody or FITC-anti murine antibody was significant to $p<0.005$. Specific antibody exhibited only background activity when incubated with control (PC only) liposomes.

Table 3 Aggregation by liposome type and addition

Liposome type	Addition	Aggregates
control liposomes	Cryoprecipitate	1+
	FGN + FNC + VWF	1+
	FNC	1+
	vWF	1+
IIb-IIIa liposomes	Cryoprecipitate	3+
	FGN + FNC + vWF	3+
	FGN + FNC	3+
	FGN	2-3+
	vWF	1+
	FNC	1+
IIb-IIIa liposomes+ Monoclonal antibody (10E5 or 7E3)	cryo	1-2+

Legend: FGN: Fibrinogen, FNC: Fibronectin, vWF: von Willebrand factor.

Table 4
Liposome stability by carboxyfluorescene release.

MEDIA:	HEPES/NaCl pH 7.4		PLASMA	
Liposome Compostion:	100% PC	PC:Chol	100% PC	PC:Chol
Day	% Release			
0	0	0	0	0
2	0	0	20	0
7	8.4	12	24.5	0
10	18	23	12.5	0
14	18	-	17.8	5.8
21	36.8	37	20	12
30	50	15	37	60
42	Not done liposome suspension totally fused.			

Legend

PC = Phosphatidylcholine

CHOL = Cholesterol

PC: CHOL = 30mole% PC:30mole% cholesterol

Table 5
Liposome Size-nm over time with plasma or buffer incubation

	Buffer		Plasma	
	PC	PC:CHOL	PC	PC:CHOL
Day				
0	4250	2620	2960	2060
2	4400	4920	2960	2060
7	12900	1120	5850	3051
10	18800/3540*	2750	2400	3510
14	14100	10800	17900/6060*	9130
21	9270/3920*	5190	18500	28100/ 7590*

Day 30 42: results invalid since liposomes totally fused.

* 2 populations of liposomes present

Table 6

Liposomes were added to plasma or whole blood and prothrombin time (PT), partial thromboplastin time (PTT) or whole blood clotting time (WBCT) performed by standard methods.

Plasma (ul)	liposomes (ul)	PT (sec)	PTT(sec)
100	0	12.0	24.6
100	20	12.0	23.0
	40	12.4	23.8
	80		23.8
	100		25.9

Whole blood clotting time

Whole blood		WBCT
500ul	0	135±10sec
500ul	20	120±12sec

n=10

no significant differences were seen among the values.

S.D pf PT and PTT±0.5 sec

TABLE 7

Effect of Liposomes on Platelet Aggregation

Agonist	Liposomes (Volume)	% Platelet Aggregation (%Light Transmission)
None		0
(spontaneous agg.)	40λ (10% v/v)	0
ADP		
1.6 uM	0	50%
1.6 uM	(5% v/v)	50%
3.2 uM	0	60%
	0-90λ (20%)	60%
Collagen	0	60%
	4%-20%	60%
Epinephrine 2.5 uM	0	50%
	10%	50%

Aggregation tracings show no change in the kinetics of aggregation.

Table 8

Liposome	Control % Adhesion	(ng lipid)	IIb-IIIa %Adhesion	(ng lipid)
Aortic Segments				
Bovine -	0.816	(84)	1.6	(505)
+ cryo	2.1	(505)	6.9	(1663)
		nsd		p<0.001
Human				
+ cryo	3.69	(885)	5.35	(1283)
				p<0.09
Preincubation of liposomes with plasma			1.4	(485)@
			@p<0.001 compared with cryo+no preincubation	
Microliter wells				
BSA		34		34.9
FGN		32		42.5*
FNC				
75 ug/ml				30.7
250 ug/ml				24.9
Collagen				
Type IV				28.2
Calf Skin				28.6

* p<0.005 compared to BSA or collagen

Table 9

Induction of thrombocytopenia is rats by external total body irradiation with a Cesium source: platelet count as a function of radiation dose.

	RADIATION DOSE				
	200	400	600	900	Control
			platelet count/ul		
Day					
4	658	484	547	407	873
10	161	227	67	19	1,184
11	486	299	107	18	1,167
12	604	250	190	19	1,044
14	984	506	548	17	1,128
17	1299	972	1293	+	1,320

\+ Rat died day 14

Table 10
Release of Trapped Glucose from
Liposomes by Sera from Rabbits and Humans

Serum source	% Glucose Release	
Normal Rabbit 1	18%	+
Normal Rabbit 2	11%	+
Normal Rabbit 3	30%	+
Normal Human 1	0%	
Normal Human 2	0%	

\+ Indicates naturally occurring
anti-lipid antibody